FREE AMINO ACIDS IN SOME AQUATIC INVERTEBRATES[1]

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Free amino acids and many of their derivatives are found present in tissues of all invertebrates so far studied. The distribution of amino acids seems to follow a pattern characteristic for the species. Compounds such as taurine were found in very high concentration in a number of invertebrates. As early as 1904, Kelly reported that *Mytilus edulis* has as much as 1.6 per cent taurine. Other invertebrates contain also large amounts of taurine (Henze, 1905; Mendel, 1904; Kossel and Edlbacher, 1915; Okuda, 1920; Ackermann *et al.*, 1924; Ackermann, 1935; Lewis, 1952; and Kermack *et al.*, 1955). The function of taurine in invertebrates is not known at all, and its mode of formation remains to be elucidated.

The distribution of free amino acids in a number of invertebrates has been studied by Camien *et al.* (1951), Duchâteau and Florkin (1954), Duchâteau *et al.* (1952), and Giordano *et al.* (1950). The amino acids were determined microbiologically. Camien *et al.* found very high concentrations of glycine in muscles of *Homarus vulgaris* and *Maia squinado* and suggested that the role of glycine along with other amino acids was to regulate osmotic pressure. Kermack, Lees and Wood (1955) made an extensive study of the non-protein constituents of the lobster. They found that a large portion of the non-protein nitrogen was accounted for as free alpha-amino nitrogen. The remainder was distributed between trimethylamine oxide, glycine betaine, taurine and volatile bases.

The object of the present study was to determine the pattern of distribution of free amino acids and related substances and to establish a correlation between the pattern of distribution and species and/or environment. The results reported here indicate that such differences do exist in relation to environment and species.

Materials and Methods

Animals investigated

The invertebrates investigated include representatives of the following phyla: Coelenterata, Arthropoda, Mollusca, and Echinodermata. The specimens were taken from their environment, rapidly frozen and maintained in this state just prior to analytic procedures (not more than a one-month period). Table I lists these organisms according to phylum and class and shows the location and habitat from which the specimens were taken.

Extraction

Immediately after thawing, the whole organism was quickly weighed and the nitrogenous substances extracted with 80 per cent ethanol according to the pro-

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[2] Part of these data was taken from the M.A. Thesis of John W. Simpson.

TABLE I

Organisms studied and their habitat

Organism*	Location of collection	Habitat
Coelenterata		
Anthozoa		
Bunodosoma cavernata	PA	W
Arthropoda		
Crustacea		
Penaeus aztecus	AB	W
Clibinarius vittatus	SLP	E
Pagurus pollicaris	AB	W
Mollusca		
Gastropoda		
Oliva sayana	SLP	E
Polinices duplicata	AB	W
Thais haemastoma haysae	AB	W
Busycon perversum	AB	W
Fasciolaria distans	PA	W
Siphonaria lineolata	PA	E
Pelecypoda		
Lithophaga bisulcata	PA	W
Crassostrea virginica	SLP	W
Arca umbonata	PA	W
Volsella demissus granosissimus	EBL	E
Cephalopoda		
Loliguncula brevis	AB	W
Echinodermata		
Holothuroidea		
Thyone sp.	SLP	W
Asteroidea		
Luidia clathrata	SLP	W

PA—Port Aransas, Texas
AB—Aransas Bay, near Rockport, Texas
SLP—San Luis Pass, Galveston Island, Texas
EBL—East Beach Lagoon, Galveston Island, Texas
W—Collected directly from marine waters
E—Collected during a period of exposure

* We are indebted to Mr. Howard Lee from the Texas Game and Fish Commission, Rockport, Texas, for permitting the use of equipment necessary for acquisition of organisms.

cedure of Awapara (1948). The amino acids extracted are not produced during the extraction procedure by proteolytic cleavage. Extractions were carried out using live organisms under conditions such as to prevent any enzymatic activity. The live organisms were ground with 80 per cent ethanol as indicated above. Others were frozen and then thawed. Extracts were also prepared as described. Analysis of the extracts revealed that no change had occurred as a result of freezing and thawing.

Fractionation of extractives

Using ion-exchange chromatography the nitrogenous extractives were separated into basic, acidic and neutral substances. Basic substances were separated from neutral and acidic substances on Amberlite CG-50, type 2 H^+, with a screen grading of approximately 200 (passing 200 mesh). The tissue extracts were placed in a beaker containing one gram of the resin and shaken for thirty minutes to allow equilibration with the resin. This suspension was placed in a small column

TABLE II

Amino acids identified

Organism	Al	B-Al	Gly	Ar	As	Glu	Tau	Gla	Pr	OH-Pr	Thr	Tyr	Aspr	His
Coelenterata														
Anthozoa														
Bunodosoma cavernata	+	−	+	+	+	+	+	+	−	−	−	−	−	−
Arthropoda														
Crustacea														
Penaeus aztecus	+	+	+	+	+	+	+	+	+	−	−	+	+	+
Clibinarius vittatus	+	−	+	+	+	+	+	+	+	−	+	+	+	+
Pagurus pollicaris	+	−	+	+	+	+	+	+	+	−	+	+	+	+
Mollusca														
Gastropoda														
Oliva sayana	+	−	+	+	+	+	+	+	−	−	−	−	−	−
Polinices duplicata	+	−	+	+	+	+	+	+	+	+	−	−	−	−
Thais haemastoma haysae	+	−	+	+	+	+	+	+	+	+	−	+	+	+
Busycon perversum	+	−	+	+	+	+	+	+	+	+	−	−	+	+
Fasciolaria distans	+	−	+	+	+	+	+	+	−	−	+	+	+	+
Siphonaria lineolata	+	−	+	+	+	+	+	+	+	+	−	+	+	−
Pelecypoda														
Lithophaga bisulcata	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Crassostrea virginica	+	+	+	+	+	+	+	+	+	−	+	−	−	−
Arca umbonata	+	+	+	+	+	+	+	+	+	−	+	−	−	+
Volsella demissus granosissimus	+	+	+	+	+	+	+	+	+	−	+	+	−	+
Cephalopoda														
Loliguncula brevis	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Echinodermata														
Holothuroidea														
Thyone sp.	+	−	+	+	+	+	+	+	−	−	−	−	−	−
Asteroidea														
Luidia clathrata	+	−	+	+	+	+	+	+	−	−	−	−	−	−

Legend:

\+ Present in readily detectable amounts
− Not present in readily detectable amounts

Al—alanine
B-Al—beta alanine
Gly—glycine
Ar—arginine
As—aspartic acid
Glu—glutamic acid
Aspr—asparagine
Tau—taurine
Gla—glutamine
Pr—proline
OH-Pr—hydroxyproline
Thr—threonine
Tyr—tyrosine
His—histidine

(20 × 1 cm.) containing an additional gram of the resin. The neutral and acidic compounds were washed from the resin with 25 cc. water and the basic substances were eluted from the column with 25 cc. 4 N HAc. (Awapara, Davis and Graham, 1959). After removal of the basic substances, taurine and other sulfonic acids were separated from the neutral and acidic amino acids on a column of Dowex-50 H^+. The water wash from the Amberlite was passed slowly through a column containing one gram of Dowex-50 H^+. Taurine was obtained by washing with 25 cc. water and the neutral and acidic amino acids eluted from the resin with 25 cc. 4 N NH_4OH. Each fraction was evaporated to dryness on a steam bath and brought to a 1-cc. volume with water.

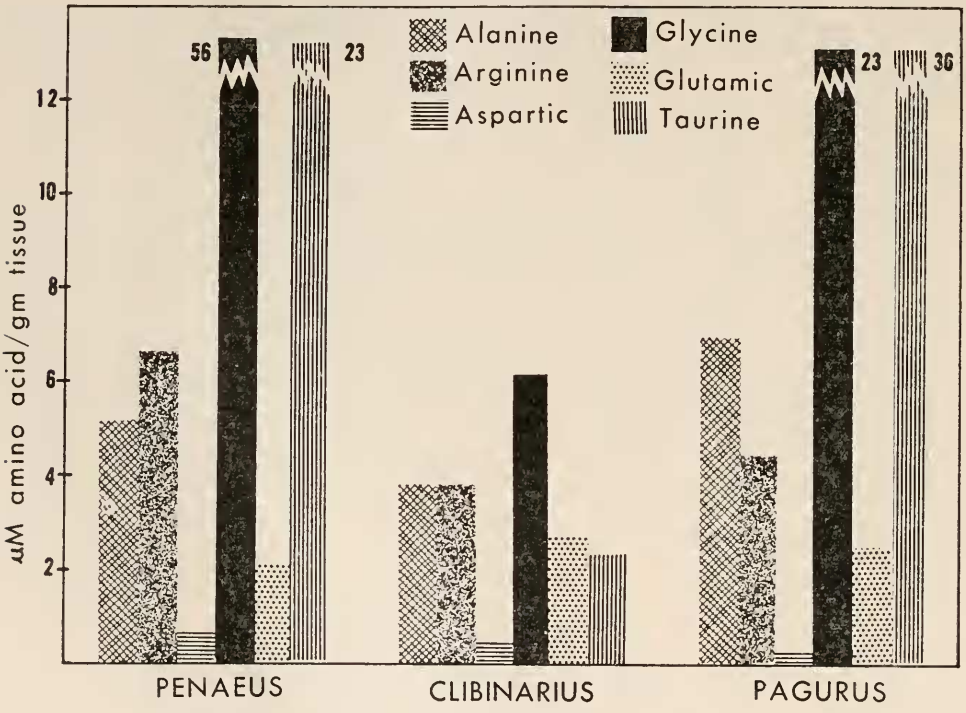

FIGURE 1. Estimated concentration of six amino acids in the tissues of three crustaceans: *Penaeus aztecus, Clibinarius vittatus* and *Pagurus pollicaris.*

Paper chromatography

Amino acids of the acidic and neutral fractions were separated by two-dimensional ascending paper partition chromatography. Whatman number 3 MM. filter paper was used and the solvent system employed was phenol-water (72.5 per cent phenol) and 2,4-lutidine-water (62 per cent) in the second direction. The basic amino acids were separated by one-dimensional ascending paper partition chromatography using butanol, acetic acid and water (4 : 1 : 1, by volume) as the solvent.

The amino acid spots were revealed by dipping the paper in a solution of 0.5 per cent ninhydrin in absolute ethanol (w/v).

Identification of amino acids

Amino acids were identified by: 1) their ninhydrin color and position on chromatograms according to previously prepared maps of known substances, and 2) their presence in a particular fraction.

Arginine was further identified by the Sakaguchi reaction, using the alpha napthol reagent described by Acher and Crocker (1952), and histidine by the Pauly's reaction using the sulfanilic acid reagent described by Smith (1958).

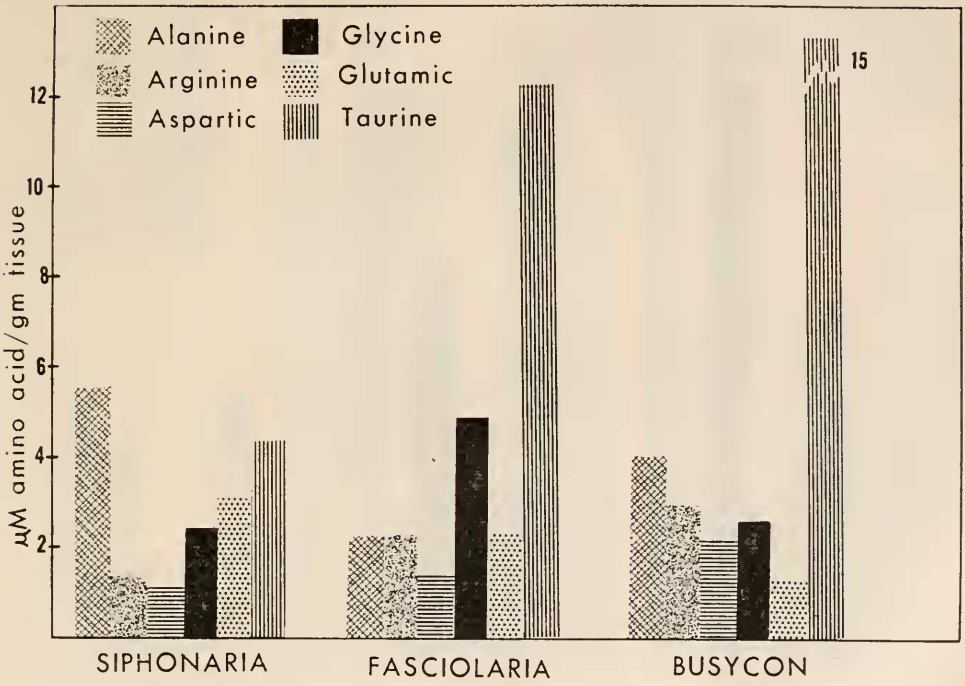

FIGURE 2. Estimated concentration of six amino acids in the tissues of three gastropods: *Siphonaria lineolata*, *Fasciolaria distans* and *Busycon perversum*.

The identification of β-alanine and taurine was supported by preparing chromatograms on paper treated with basic cupric carbonate (Block *et al.*, 1958). These two substances moved to their respective positions on both treated and untreated papers.

Glutamine was further identified by subjecting an aliquot of the extract to hydrolysis with 6 N HCl. Chromatograms of the hydrolysed extracts showed the disappearance of this spot; further, these same chromatograms showed an increase in the glutamic acid spot.

The identification of the asparagine spot was substantiated by elution from undeveloped chromatograms with subsequent acid hydrolysis and re-chromatography of the eluate. Chromatograms of the hydrolysate showed the absence of the spot and the appearance of a spot which corresponded in position and color with the aspartic acid standard.

Estimation of amino acids

Six amino acids which were consistently present were estimated quantitatively. Alanine, aspartic acid, glycine and glutamic acid were measured by the method of Awapara, Landua and Fuerst (1950) with the following modifications suggested

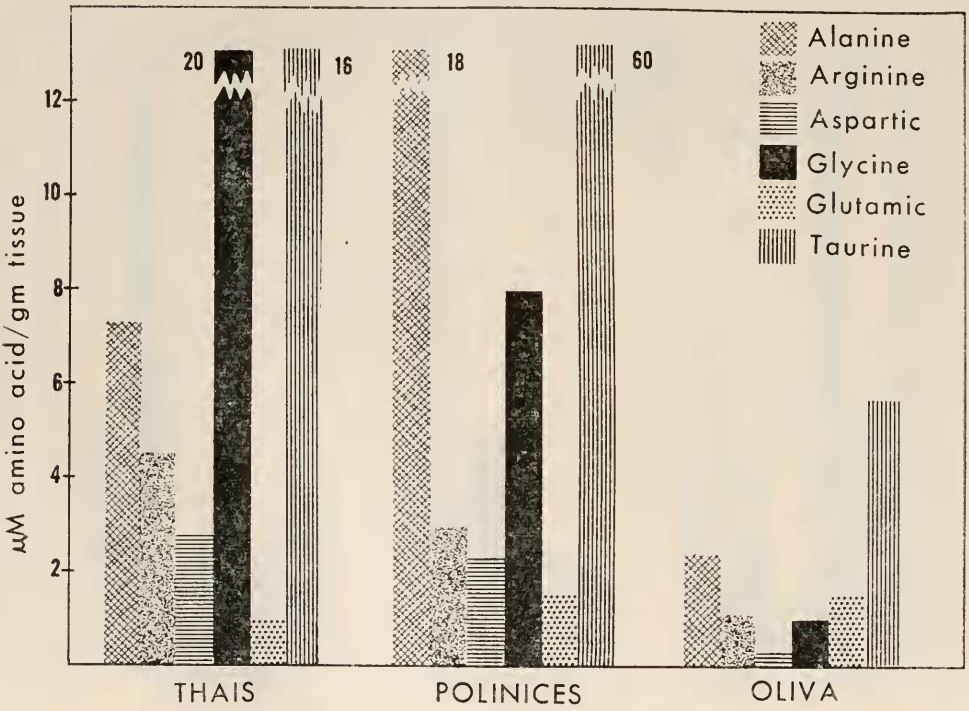

FIGURE 3. Estimated concentration of six amino acids in the tissues of three gastropods: *Thais haemastoma, Polinices duplicata* and *Oliva sayana.*

by Fowden (1951): 1) the areas of paper containing the spots were cut, placed in test tubes, and 0.2 cc. N NaOH added. The tubes were evacuated for three hours in a desiccator containing concentrated H_2SO_4; 2) additional citrate was included in the ninhydrin reagent of Moore and Stein (1948) equivalent to the amount of NaOH added.

Taurine was found to be the only ninhydrin-positive material present in the acidic fraction; therefore, this substance was measured directly using the color reagent of Moore and Stein (1948).

Arginine was measured in the extracts by the method of Rosenberg *et al.* (1956). We found no other Sakaguchi-positive material in the extracts as determined by paper chromatography. If other guanidine derivatives were present, their concentration was too low to measure. We can safely assume that nearly all the color in the Rosenberg reaction was due to arginine .

Results and Discussion

In Table II are shown all the amino acids and related substances detected chromatographically. Other components were present but their identity was not established. In Figures 1 to 6 are shown the concentrations of six amino acids

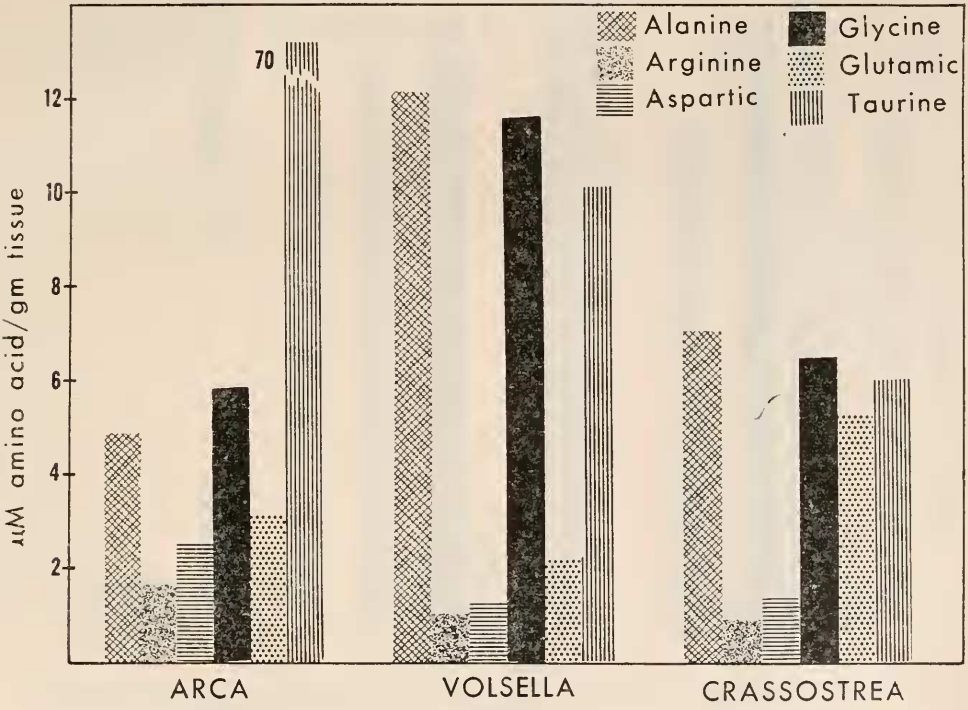

Figure 4. Estimated concentration of six amino acids in the tissues of three pelecypods: *Arca umbonata, Volsella demissus granosissimus* and *Crassostrea virginica.*

which were found invariably in the species studied. The most striking feature is the high concentration of taurine and glycine in nearly all species studied. The glycine concentration is in many cases equal to that of taurine. The highest concentration of taurine was found in *Penaeus aztecus,* but our value is still considerably lower than the value reported for *Mytilus edulis.* The mode of formation of taurine in invertebrates is not known at all. In mammals it is formed from cysteine after oxidation to cysteine sulfinic acid and decarboxylation of the latter

to hypotaurine (Awapara and Wingo, 1953). Hypotaurine is then oxidized to taurine. Hypotaurine has been reported only once in invertebrates (Shibuya and Ouchi, 1957). The cysteine sulfinic decarboxylase has not been reported in invertebrates. There is the possibility that taurine is not produced by invertebrates but acquired from their diet. Glycine, which is also present in very high amounts, could also be obtained from their diet. One objection to this possibility is the wide range of variation in the taurine concentration of species from the same environment.

Aspartic acid, glutamic acid, and alanine vary much less in concentration than taurine and glycine.These three amino acids are closely linked to the citric acid

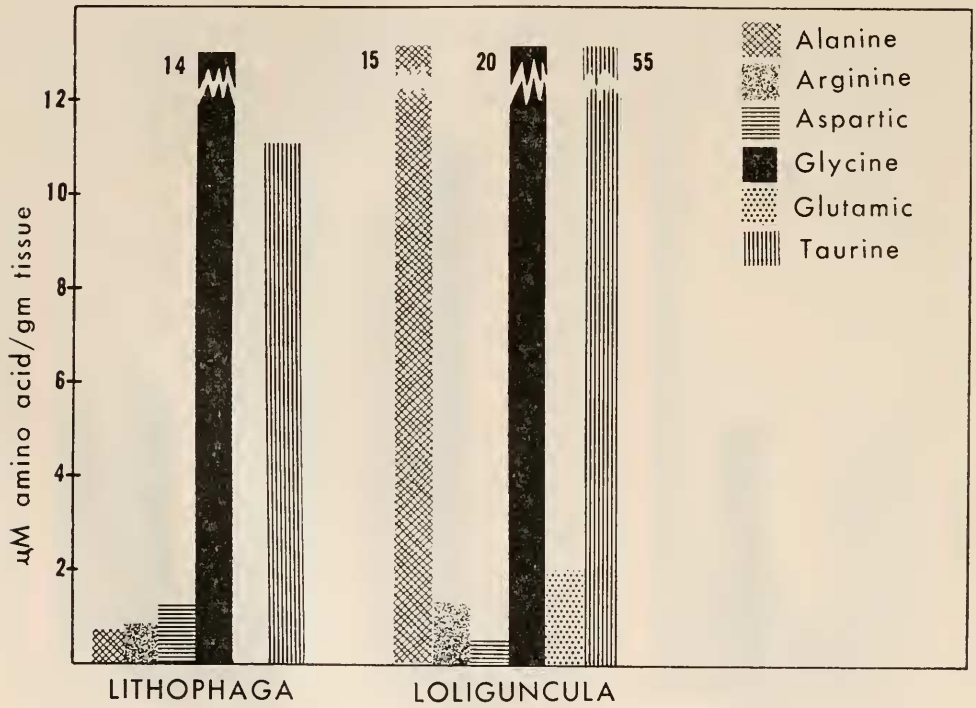

FIGURE 5. Estimated concentration of six amino acids in the tissues of pelecypods *Lithophage bisulcata* and the cephalopod *Loliguncula brevis*.

cycle and their concentration could be easily regulated by the reactions of the cycle. β-alanine was found in some forms but not in others. The only coelenterate which we studied had none. It was found in some Mollusca but not in others. The same was true in the arthropods studied. The function of β-alanine is again unknown. It exists usually as a moiety of carnosine which is known to exist in the muscle of vertebrates and many invertebrates. The problem of formation arises again. β-alanine can be produced by the decarboxylation of aspartic acid, or by the hydrolytic breakdown of dihydrouracil. Similarly, β-aminoisobutyric acid is formed by the hydrolytic breakdown of dihydrothymine. We found β-aminoisobutyric acid in

small amounts in *Volcella* and in larger amounts in *Mytilus*. A somewhat curious finding is that of asparagine in certain representatives of the Mollusca and Arthropoda, as shown in Table II. Asparagine is not often found in detectable amounts in mammals.

Arginine was present in all the species studied. It probably resulted from the hydrolysis of arginine phosphate, a well-known phosphagen in invertebrates.

Inasmuch as the taurine concentration appeared to vary with the environment we proceeded to study a number of fresh-water forms and also other marine forms for their taurine content. The results are shown in Table III. The presence of

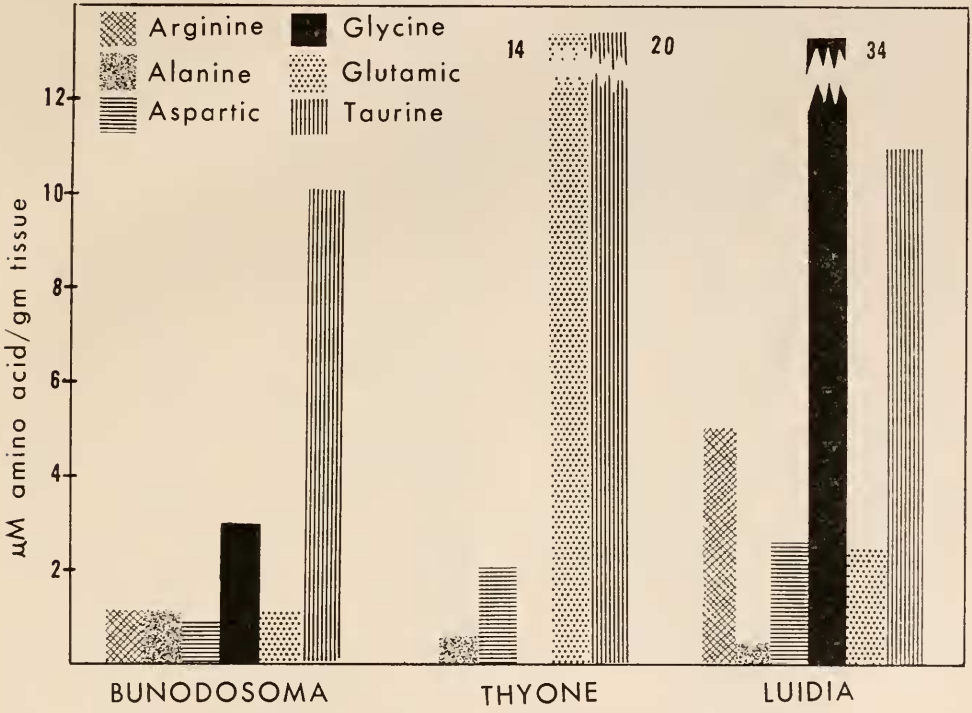

FIGURE 6. Estimated concentration of six amino acids in the tissues of the coelenterate *Bunodosoma cavernata* and the echinoderms *Thyone* sp. and *Luidia clathrata*.

taurine by chromatography is detectable in concentrations as low as 0.1 micromole per gram. Our results indicate that taurine is either absent in the fresh-water forms of molluscs or it is present in concentrations lower than 0.1 micromole per gram. Every fresh-water and terrestrial mollusc studied showed no taurine whereas every form of invertebrate living in salt water or brackish water contained taurine in detectable and measurable amounts.

At this stage of this study, it is not possible to attribute to taurine any definite role. But the evidence is suggestive that it plays a very important role as an osmoregulator.

TABLE III

Taurine content in various molluscs

Mollusca	Environment	Taurine
Gastropoda		
Lymnaea palustris	Fresh water	−
Marisa cornuarietis	Fresh water	−
Pomacea bridgesi	Fresh water	−
Rumina decollata	Terrestrial	−
Otala lactea	Terrestrial	−
Mesodon thyroidus	Terrestrial	−
Bulimulus alternatus	Terrestrial	−
Murex fulvescens	Marine	+
Littorina irrorata	Marine	+
Oliva sayana	Marine	+
Polinices duplicata	Marine	+
Busycon perversum	Marine	+
Siphonaria lineolata	Marine	+
Fasciolaria distans	Marine	+
Thais haemastoma haysae	Marine	+
Pelecypoda		
Anadonta grandis	Fresh water	−
Quadrula quadrula	Fresh water	−
Lampsilis sp.	Fresh water	−
Elliptio sp.	Fresh water	−
Rangia cuneata	Brackish-Fresh water	+
Brachiodontes recurvus	Brackish-Marine	+
Crassostrea virginica	Brackish-Marine	+
Donax variabilis	Marine	+
Venus mercenaria	Marine	+
Dosinia discus	Marine	+
Arca incongrua	Marine	+
Arca campechiensis	Marine	+
Noetia ponderosa	Marine	+
Cephalopoda		
Loliguncula brevis	Marine	+

SUMMARY

1. Free amino acids of 17 species of aquatic invertebrates were determined by chromatographic methods.

2. Qualitative differences in some amino acids were detected in organisms of different species. The concentration of alanine, arginine, aspartic acid, glutamic acid, glycine and taurine was measured and significant differences recorded.

3. Taurine was found in high concentration in all the marine organisms studied but was not found in several fresh-water and terrestrial organisms.

4. The possible role of taurine and other free amino acids in aquatic organisms is discussed.

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